Ribosomal Intergenic Spacer- and DGGE- based Analyses of Microbial Consortia Associated with Liquid and Particulate Fractions of Rumen Digesta

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Abstract

It is well recognized that ruminal microbes develop a dynamic biofilm upon digesta particles, with some bacteria tightly adherent and others more loosely associated. However, rumen microbial diversity is commonly examined using only liquid-based fractions. To assess the biodiversity associated with the biofilms present on digesta particles, the rumen contents from four sheep, fed a diet either entirely of grass hay, or a combination of corn and grass hay (70:30), were separated into three fractions: liquid (strained through cheesecloth), associated (bacteria recovered by washing particles with buffer at room temperature) and adherent (bacteria extracted with buffer containing 0.15% v/v Tween-80, and chilling). The genomic DNA from sub samples of these communities was extracted and then subjected to either RIS (Ribosomal Intergenic Spacer) analysis, or DGGE analysis of the V3-region of the 16S rRNA gene. Of the two methods, the RIS profiles appeared to provide the most diverse banding patterns with respect to both diet and fraction of digesta. The RIS-PCR products generated from the four adherent communities were then cloned and subjected to RFLP analysis. The resulting patterns provided further evidence that the adherent communities of the four animals were affected by exogenous (diet) and endogenous (host derived) parameters. Clones obtained from each adherent community were randomly selected and subjected to DNA sequence analysis. Most of the sequenced clones obtained from animals consuming an all-grass diet appear to be most similar to *Clostridium*, *Prevotella* or *Selenomonas* species, but the sequence identity is less than 95% in most instances. From

the animals consuming a grain-based diet, the sequenced clones are most similar to the *Ruminococcus, Selenomonas*, and *Mitsuokella*. Most libraries were less than 25 percent of the sequenced clones belong to the Prevotella/Bactericides subgroups, which are the numerically dominant sequences in clone libraries prepared from whole digesta. The results reveal a relatively large population of uncharacterized bacteria potentially involved in polysaccharide degradation.

1. **Introduction**

Microbial fermentation in ruminants involves a complex group of unique microorganisms, which includes bacteria, fungi, and protozoa (Flint, 1997). It is through these microorganisms that ruminants are able to utilize high forage diets and break down the cellulose and other polysaccharides present in the plant cell wall (hereafter referred to as fiber). The hydrolytic and fermentative activities of the microorganisms not only supply the animal with volatile fatty acids, i.e. energy, but also vitamins and microbial protein. Many of these microorganisms are obligate anaerobes, and there are both cooperative and competitive interactions among species that influence the kinetics of fiber hydrolysis and fermentation. The traditional way of investigating the biodiversity of rumen microorganisms has been to try to culture and isolate various species, but it is difficult to culture and maintain many of these microorganisms. There is a need to improve the breadth of profiling and examination of the genetic diversity of rumen microorganisms, beyond the microbes that can be cultivated in the lab.

DNA-based analysis does not require cultivation of the bacteria from an environmental sample prior to community structure analysis. Sedatives such as the 16S ribosomal DNA gene (rDNA) are used to study both relationships and speciation among bacteria. The 16S rDNA gene is considered as a useful molecular tool in such studies, because it meets many criteria necessary to examine bacterial relatedness and diversity. The 16S rDNA gene encodes the 16S rRNA, which along with ribosomal proteins; assemble to form the

small subunit of the ribosome. Accordingly, the 16S rDNA gene encodes an essential cellular function, and a version of this gene is present in all known organisms. Secondly, it contains regions of both conservation and divergence, allowing similarity to be used as a measure of relatedness. Thirdly, the molecule is comprised of ~1600 nucleotides, the sequence of which can be obtained quickly and cheaply. Therefore it is both large enough to allow powerful comparisons, but small enough that today's technologies support its rapid acquisition. Microbial diversity and community structure is now commonly examined by the creation of 16S rDNA clone libraries and DNA sequencing. A clone library is created by cloning a specific region of DNA from all microbes present in environment samplings, after its amplification by a method called polymerase chain reaction (PCR). The PCR products should provides an accurate representation of the diversity present in the microbial community that was sampled. The PCR products are inserted into plasmids and propagated in *E. coli* which is easily grown and maintained in the lab.

Although these methods have expanded our ability to more completely annotate microbial diversity, a shortfall with 16S clone libraries is that there is not always enough sequence diversity to distinguish clearly among species within the same genus (Yu et al., 2001). This is an important concern in studies that want to annotate a more complete representation of the diversity of a community, where different species of the same genus can carry out vastly different function or efficiencies of utilization. In eubacteria, the intergenic region between the 16S and 23S rRNA genes is highly divergent, especially in terms of its length. Ribosomal Intergenic Spacer Analysis (RISA) has become an

attractive method to analyze microbial diversity, because the resulting RIS amplification products can be compared in terms of length polymorphisms (RIS-LP) as well as by restriction fragment length polymorphisms (RIS-RFLP) (Ranjard et al., 2001 and Toth et al., 2001). Moreover, because the primers used for RISA provide a substantial amount of 16S rRNA gene sequence, the products can still be used for species identification.

There is an interest in the microbial diversity associated with plant cell wall hydrolysis in anaerobic environments, because the processes are central to carbon recycling and sequestration, as well as more pragmatic issues, such as forage digestion in cattle, landfill reclamation, and the production of solvents and alcohols from plant biomass. The rumen is one such environment, and the rates and extent of polysaccharide hydrolysis are relatively rapid. Although several 16S rDNA clone libraries of rumen microbes have been constructed and analyzed, they were created with liquid fractions of digesta, which are dominated by clones representing the *Prevotella* and *Bacteroides* genera (Ramsak et al., 2000). However, relatively little effort has been made to examine the microbial diversity that resides within discrete regions of this microbial community. It is well recognized that ruminal microbes develop a dynamic biofilm upon plant particles, with some bacteria tightly adherent and others more loosely associated. These adherent populations are believed to be dominated by microbes critical to the hydrolysis of plant polysaccharides, releasing soluble carbohydrates for use by other members of the microbial community.

I hypothesized that the microbial diversity will differ with respect to the sampling site (liquid vs. adherent populations), as well as in response to animal diet (an all grass hay vs. a concentrate: hay diet) and these differences will be detected by DNA-based methods of analysis. To address this hypothesis, I have used a combination of DNA- based methods to examine the microbial diversity in different fractions of rumen digesta. Microbial diversity was examined with both DGGE (denaturing gradient gel electrophoresis) and RIS (ribosomal intergenic spacer) analysis. RIS products were then subjected to a double digest with Alu I and Hae III, and unique phylotypes were sequenced. The information was subjected to both statistical and other forms of *in silico* analysis to compare and contrast the microbial communities present in the different samples.

2. **Materials and Methods**

Animals and diets. Four ruminally cannulated sheep were maintained at the OARDC research facilities in Wooster, Ohio, according to procedures advocated by Ohio Animal Care and Use Committee. Two animals were fed (once daily) a diet of orchard grass hay (called H1 and H2), and two others were fed a diet of whole corn and orchard grass hay, at a 70:30 ratio (called C1 and C2).

Sample collection and fractionation procedures. Ruminal samples were collected 6 hours post-feeding through the rumen cannula and transferred to a sterile beaker. The ruminal digesta was taken to the laboratory and separated into three fractions: liquid, associated and adherent (see Figure 1). The liquid fraction, containing mainly the free-floating bacteria, was prepared by squeezing 250 to 1000 ml of digesta through two layers of cheesecloth. The liquid was subdivided into 150 ml aliquots and centrifuged at 4C for 20 minutes at 10,000 x g. The supernatant fraction was removed and the bacterial pellet was resuspended in 50 ml of TE buffer. These microbes are considered to represent the liquid fraction of the digesta. Next, the solids that were retained in the cheesecloth were processed. A sub sample (50 grams) was resuspended in 150 ml of phosphate buffered saline and mixed by shaking for approximately 30 seconds. The mixture was transferred to large clean bottles and centrifuged at room temperature for 15 minutes at 350 x g. The supernatant fraction was carefully removed and transferred to new bottles, and centrifuged again at high speed. (20 minutes at 4C and 10,000 x g). The resulting pellet was resuspended in TE buffer and considered to contain an enriched fraction of the

bacteria that are not tightly bound to plant particles, but are associated with this material more so than the liquid (hereafter referred to as the associated bacterial fraction). Finally, the plant particles recovered after low speed centrifugation were suspended in 25 ml of an anaerobically prepared dilution buffer, to which Tween 80 had been added to give a final concentration of 0.15% (vol/vol). The mixture was placed on ice for 2.5 hours, to elute the tightly adherent bacteria according to the methods of Dehority (1996), and the mixtures were then centrifuged at room temperature at 500 g for 15 minutes. The supernatant; containing adherent bacteria, which were recovered by centrifugation (4C, 10,000g, 20 min.) to separate the microorganisms into a pellet. The pellet was then resuspended in 10 ml of TE. In total, 24 fractions have been prepared (4 animals X 2 diets X 3 fractions per sample).

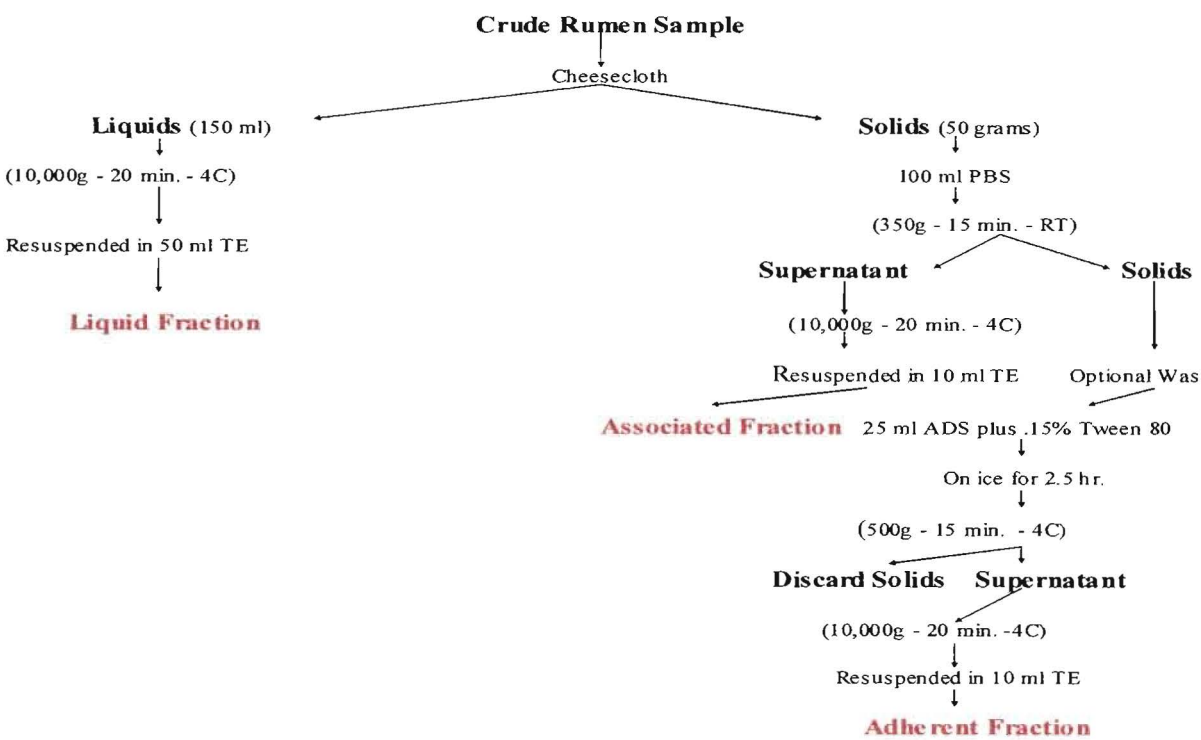


Figure 1. The procedures used to fractionate the rumen microbes into liquid, associated and adherent fractions.

DNA extraction procedures. The community genomic DNA of all fractions was extracted through a combination of bead beating and the "Two Bird" method developed by Yu and Mohn (2001). Briefly, the cells were disrupted by bead beating. Zirconium beads (0.3g of 0.1 mm and 0.1 g of 0.1 mm beads) were mixed with 1 ml of bacterial sample and agitated for 3 min. The mixture was incubated for 15 min. at 70C and then ammonium acetate was added to precipitate proteins. After centrifugation, the supernatant was recovered and the nucleic acids were precipitated with isopropanol. The resulting pellet was resuspended in TE buffer and both RNAse A and proteinase K were added to digest RNA and proteins, respectively. After enzyme digestion, the DNA was recovered by column purification using QIAamp column from the Qiagen stool kit according to manufacture recommendations (Qiagen). The concentration of DNA recovered was determined spectrophotmetrically.

RIS- and DGGE-analyses. The RIS regions of eubacterial DNA were amplified by PCR, using primers 926f and L189r, and 100 ng of genomic DNA. The thermal cycling conditions were: 94C for 45 sec (denaturing), 47C for 45 sec (annealing), and 72C for 3 min (extension), for a total of 32 cycles. The PCR products were subjected to PAGE using 3.5%T gels (37.5:1) and stained with GelStar. The images were recorded using a ChemiImager 6600 (Alpha Innotech) and analyzed using Bionumerics software.

Microbial diversity was also examined by Denaturing Gradient Gel Electrophoresis (DGGE) of the V3-region from eubacterial 16S rRNA genes. The V3-region was amplified using primers 357f and 519r, and the following thermal cycling conditions: –

94C for 40 sec (denaturing), 58C for 45 sec (annealing) and 72C for 1 min (extension) for a total 31 cycles. The products were then resolved on 40-70% DGGE gels (Muyzer et al, 1998, 100% denaturant being 40% (vol/vol) formamide and 7M urea). Gel staining, image acquisition, and analysis were performed as described above.

Construction of RIS-libraries. The PCR products generated from the Liquid and Adherent fractions were cloned, using the TOPO-cloning vector (Invitrogen) and electrocomponent *E. coli.* From these 8 libraries, a total of 768 clones were confirmed to contain insert DNA, by PCR using M13f and M13r primers and gel electrophoresis. Next, a second aliquot of the PCR products was digested with both *Hae* III and *Alu* I and the digested DNA was subjected to agarose gel electrophoresis. A total of 321 unique RFLP (restriction fragment length polymorphism) were identified, and each was considered to be its own phylotype.). Each unique phylotype was sequenced at the Plant Microbe Genomic Facility at The Ohio State University, using 1527r primer and column purified PCR products. PCR products that did not provide adequate read lengths were resequenced in purified plasmids. Dendogram comparisons were created of each library and the entire sequences.

Statistical analyses. The RFLPs generated from each library were compared using BioNumerics software, and rarefaction analysis was also performed to evaluate microbial diversity Total diversity was predicted using a combination of a linear curve and monomolecular curves (where the monomolecular curve is equal to - Number of Phylotypes = Asymptote (1- Beta * e^ (k * # of individuals))). A manual break was

assigned at the point where the curve is switched from linear to monomolecular so the number of unique phylotypes did not exceed the total species. This would occur if only a monomolecular curve is used. The Shannon-Weaver index, richness, evenness and equitability of the libraries were calculated using the methods described by Atlas et al (Atlas et al., 1993)

- Richness [d = (# phylotypes –1)/(log N)]
- Shannon-Weaver [H = 2.3/N(NlogN -$\sum$NilogNi)]
- Evenness [e = H/log # phylotypes]
- Equitability [J = H/Hmax]
    - (N = number of individuals and Ni = number of individuals per ith phylotype)

3. Results

The RIS-LP and DGGE profiles obtained from the 12 samples are shown in Figures 2 and 3, respectively. The profiles could be separated into distinct clusters on the basis of diet, although each type of analysis resulted in a different ordering. In most cases, the adherent (Ad) and associated (As) fractions from the same animal were found to be most similar, by both types of analysis. However, the RIS-LP appeared to order the fractions with respect to diet *and* animal, suggesting there was only a limited difference among the fractions. Due to the similarity of the Ad and As fractions and time constraints, the rest of the comparisons were made between the adherent and liquid fractions. As the RIS-LP provided a better separation, as well as more sequence data, subsequent analyses were done using the RIS-LP cloned products.

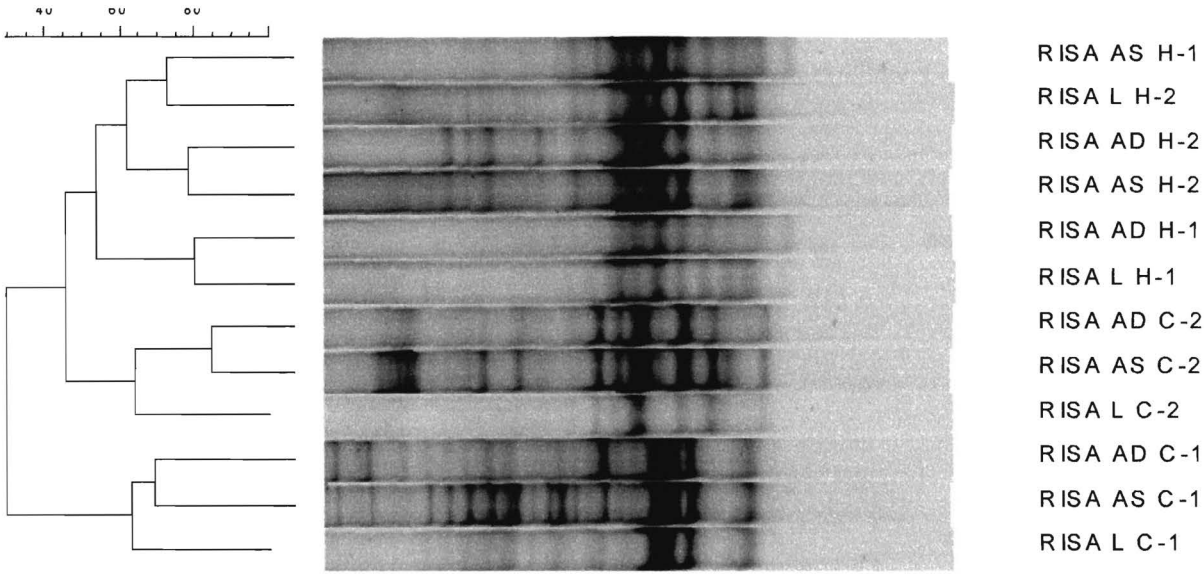


Figure 2. RIS-LP profiles and dendogram analysis of fractionated rumen digesta samples collected from sheep fed either a grass hay (H-1 and H-2) or grain: hay (70:30, C-1 and C-2) ration. Rumen microbes were fractionated into liquid (L), associated (AS) and adherent (AD) subpopulations, as described in the materials and methods.

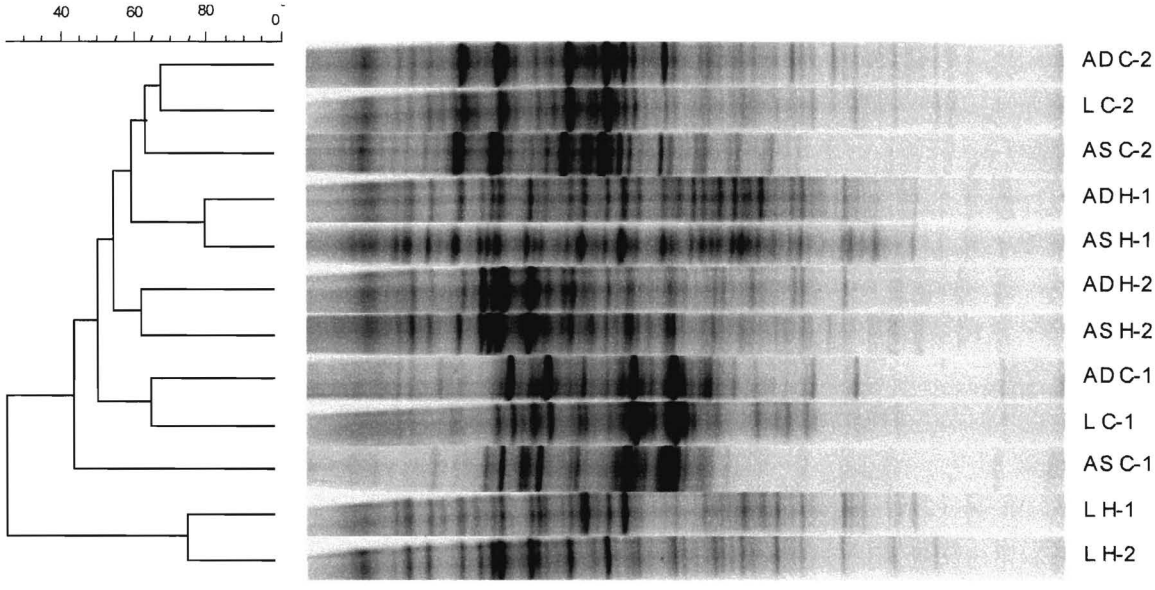
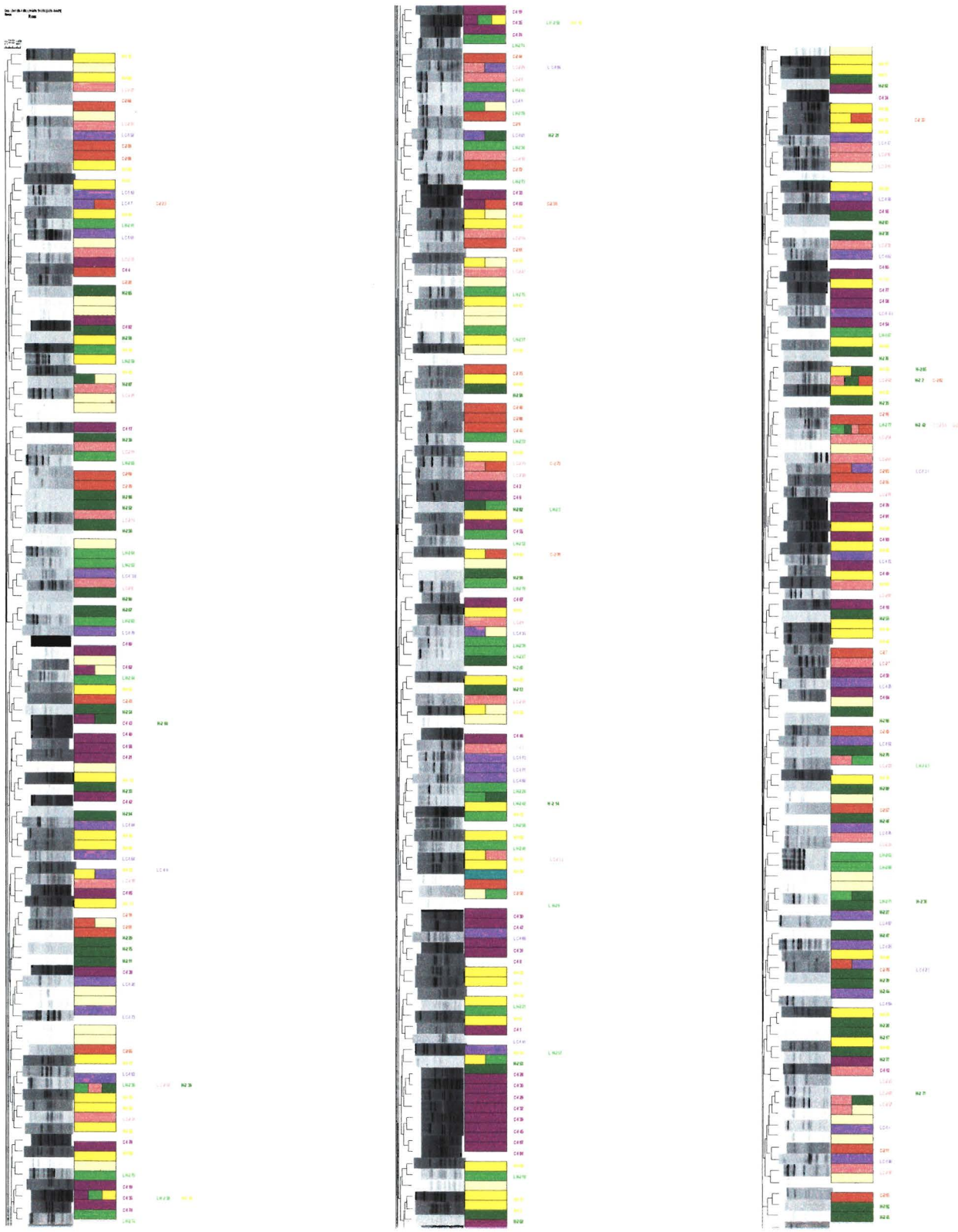


Figure 3. DGGE profiles and dendogram analysis of fractionated rumen digesta samples collected from sheep fed either grass hay (H) or grain: hay (70:30, C) ration. Rumen microbes were fractionated into liquid (L), associated (AS) and adherent (AD) subpopulations, as described in the materials and methods.

The RIS products were subjected to double digests for further identification of unique clones. From a total of 762 clones, 321 unique RFLPs were identified (see Figure 4 for the complete dendogram and Appendix A for dendograms of each of the fractions). This is somewhat in contrast to the results obtained by RIS-LP, which suggested there was relatively little microbial diversity present in the samples, based on the number of LP that could be resolved in by PAGE analysis of the RIS-PCR products.

Figure 4. This shows the entire dendogram of 321 phylotypes, as well as a color representation: Ad C-1 dark purple, L C-1 light purple, Ad C-2 red, L C-2 pink, Ad H-1 yellow, L H-1 tan, Ad H-2 dark green, and L H-2 green.



Rarefaction analysis also confirmed there was a large degree of microbial diversity present in the different samples. Diversity was predicted to be greatest for the adherent population of hay-fed animals and least in liquid fractions obtained from concentrate-fed animals based on rarefaction analysis (See Figure 5).

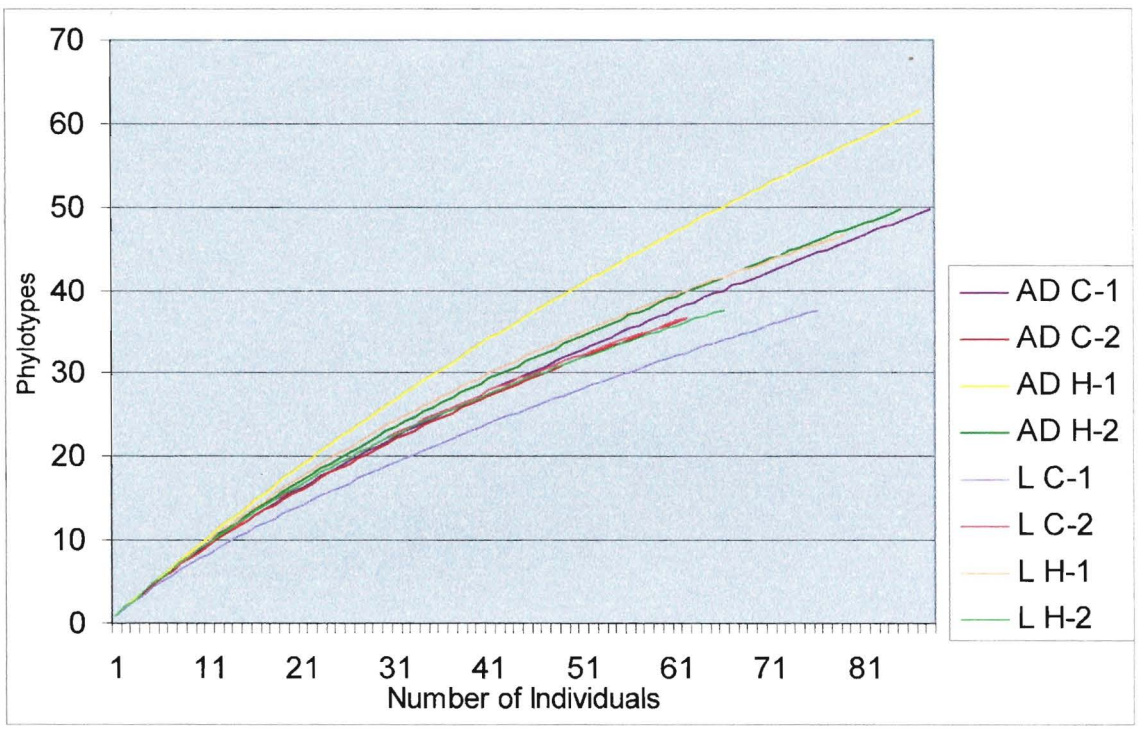


Figure 5. Graphical representation of the rarefaction analysis curves for the microbial subpopulations present in the adherent (AD) and liquid (L) fractions of rumen digesta, collected from sheep fed either all hay (H-1 and H-2) or grain: hay (70:30) rations (C-1 and C-2).

Using a combination of linear and monomolecular curves, the rarefaction analysis was also used to predict the total number of species (see Table 1). Similar results were seen

as with the rarefaction; however the coverage of the predicted diversity was closer to 50 percent.

Table 1. The predicted number of species to be identified in the digesta samples (as previously defined), and the predicted number of clones needed to provide 50% and 99% coverage, based on the rarefaction analysis.

	Rarefraction Analyses Colones Screened		
Fraction	Predicted RFLPs	Clones Screened for 50%/99%	
AD C-1	101	91	621
AD C-2	67	55	370
AD H-1	126	90	603
AD H-2	88	72	485
L C-1	72	72	493
L C-2	58	44	296
L H-1	72	53	359
L H-2	58	44	296

Like the rarefaction analyses microbial diversity, measured by Shannon-Weaver index, richness, evenness, and equitability is predicted to be greatest within the Adherent -hay fractions and least in the Liquid -concentrate fractions (see Table 2). The Shannon-Weaver index is a ratio of the number of clones minus the individuals per phylotype: that is as there is more individuals per phylotype, the number gets smaller and population is predominated by a few phylotypes. Richness is ration of phylotypes to clones: more unique phylotypes per clones screened, the higher the number the more diversity present. Equitability is the closeness to maximum Shannon-Weaver: the higher the number the

more diversity present. The ratio of the Shannon-Weaver index to number of phylotypes: the larger the number the more even and uniform the community.

Table 2. The four measures of microbial diversity predicted by Shannon-Weaver index, richness, equitability, and evenness, respectively. Diversity was predicted to be highest for AD H-1 and lowest for L C-1.

Diversity Present in the Libraries

Fraction	# Clones	Unique Clones	Richness	Shannon-Weaver	Evenness	Shannon Max	Equitability
AD C-1	89	50	25.14	3.56	2.10	4.48	0.796
AD C-2	63	37	20.01	3.34	2.13	4.14	0.807
AD H-1	88	62	**31.37**	**4.00**	**2.23**	4.47	**0.895**
AD H-2	86	50	25.33	3.65	2.15	4.45	0.829
L C-1	77	38	**19.61**	**3.13**	**1.98**	4.34	**0.721**
L C-2	63	37	20.00	3.41	2.17	4.14	0.824
L H-1	80	47	24.17	3.67	2.19	4.38	0.838
L H-2	67	38	20.26	3.44	2.18	4.20	0.819

Characterization of RIS-libraries by 16S rDNA gene sequence analysis. The complete list of microbes identified in the RIS libraries prepared from liquid and adherent fractions of rumen digesta samples are shown in Appendix B. Of the 317 satisfactory sequences obtained, only 20 were >98% identical, and 82 were >95-98% identical to sequences obtained from known bacteria, allowing a strong prediction of these clones. The remainder of the clones fell into two categories: many of the clones (201) were found to possess their highest degree of sequence identity, >90% identity, with sequences deposited in the databases for "unidentified/uncultured" bacteria and the closest known

species match was less than 95%. The second category (16) includes those sequences that had a relatively low level of sequence identity, <90%, with sequences, either known or unculturable, in the database. Therefore, clones falling into these two categories also list the best match with cultured organisms as well, see Appendix B. A comparison of the species composition represented in the different libraries is shown in Table 5.

Table 5.

Table 5: Comparisons of all sequences								
	Percentage of total clones sequenced							
	AD C-1	AD C-2	AD H-1	AD H-2	L C-1	L C-2	L H-1	L H-2
Prevotella	4	29.7	25.8	18	21.1	18.9	25.5	13.2
Clostridium	4	16.2	27.4	26	13.2	24.3	17	21.1
Selenomonas	18	29.7	4.8	10	21.1	21.6	25.5	23.7
Butyrivibrio	14			6	15.8	1.6	2.1	
Ruminococcus	20	13.5	3.2	8	2.6	8.1	2.1	5.3
Mitsuokella	12	2.7			7.9		2.1	5.3
Bacteroides	4	2.7	8.1	2	2.6		2.1	7.4
Eubacterium	2	2.7	1.6	2	2.6	2.7	2.1	2.6
Anaerovibrio	8			2	5.3	2.7	2.1	
Papillbacter	2		4.8	2			2.1	5.3
Firmicutes			4.8	4		2.7		
Morella			6.5		2.6			2.6
Lactobacillus		2.7	1.6	2			6.4	
Pseudomonas	2						2.1	
Green-nonsulfur			1.6	2		2.7		
Mogibacterium			1.6			2.7		
Methanogens	4				5.3			
Cytophagales				2				2.6
Flavobacteriaceace	2		1.6					
	Percentage of total RFLPs represented in each library							
	AD C-1	AD C-2	AD H-1	AD H-2	L C-1	L C-2	L H-1	L H-2
Prevotella	9	35	26.1	20.9	46.8	22.2	30	20.9
Clostridium	2.2	27	22.7	33.7	9.1	15.9	23.8	13.4
Selenomonas	15.7	30.1	5.7	8.1	15.6	17.5	22.5	22.4
Butyrivibrio	25.8			11.6	7.8	1.6	2.5	
Ruminococcus	14.6	7.9	3.4	9.3	1.3	7.9	1.3	3
Mitsuokella	9	1.6			9.1		1.3	3
Bacteroides	3.3	1.6	8	1.2	1.3		2.5	4.5
Eubacterium	1.1	1.6	1.1	1.2	1.3	3.2	2.5	1.5
Anaerovibrio	5.6			1.2	2.6	1.6	1.3	
Papillbacter	1.1		4.5	1.2			1.3	3

Firmicutes			3.4	2.3		1.3		4.5
Morella			11.4		6.5			
Lactobacillus		1.6	1.1	1.2			5	
Pseudomonas	1.1						4.5	
Green-nonsulfur			1.1	1.2		4.8		
Mogibacterium			1.1			1.3		
Methanogens	2.2				2.5			
Cytophagales				2.2				1.5
Flavobacteriaceace	5.6		1.1					

Footnote: The first set is the percent that species represents out of the sequences. The second set the percent of occurrences of that RFLP in the total library. Where the totals due not add up to 100% it is due to occurrence of species that did not appear in more than one fraction and it was not included in the table (but was in the calculations).

In the Adherent C-1 library, clones most identical to *Ruminococcus* and *Selenomonas* were the most numerous in terms of DNA sequences, coupled with a lot of diversity in RIS-RFLPs. However, clones matching *Butyrivibrio* sequences were the most numerous in the library although the RIS-RFLPs obtained for these sequences were limited in diversity. The Adherent C-2 library contained the greatest percentage of *Prevotella* and *Selenomonas* like sequences and was similar to the Liquid C-1 library in this regard. The two libraries prepared from the adherent populations of hay-fed animals were very similar to each other. Both contained a relatively large percentage of sequences identified as being most identical with *Prevotella* and *Clostridium* spp. and with higher diversity of RIS-RFLP patterns for the AD H-1 library. The libraries prepared from the liquid fractions of C-2, H-1 and H-2 was largely comprised of sequences most closely similar to Prevotella, *Clostridium* and *Selenomonas* spp.

There were also a series of clones that were identified infrequently, or only in one animal, and these include Cytophaga and *Papillibacter*. Surprisingly, one or two clones also appeared to be most identical to methanogens. Being archaea, these clones were not expected to arise in the libraries because of the primer specificity.

1. Discussion

Tajima et al. (2001) monitored by real time PCR the populations of 13 common rumen species during a switch from hay to a grain based diet. Similarly, my use of DGGE and RISA also separated the fractions/animals with respect to diet.

However, the RIS-LP profiles clustered with respect to both diet *and* animal, suggesting that microbial diversity was also similar between the fractions of ruminal digesta, and that little diversity existed among the different samples. Such a suggestion is contradicted though by the RIS-RFLP analysis, and in fact, the statistical analyses indicate the bacterial population adherent to plant material is not only different to the liquid fractions, but are also more diverse than those recovered from liquid. This provides further support for the additional step of RIS-RFLP, as studies based only on RIS-LP do not fully account for the biodiversity present in the samples.

The rarefaction analysis also indicates we have not obtained a full representation of microbial diversity from each of these fractions. We estimate that up to 620 clones are required for the libraries to approach saturation. Indeed, the sequenced clones lack some

of the most common rumen bacterial groups, notably *Fibrobacter* spp., especially *Fibrobacter succinogenes*. Some studies have estimated *Fibrobacter succinogenes* is more numerous than *Ruminococcus albus* or *Ruminococcus flavefaciens*, which are generally regarded as the other two main cellulolytic bacteria (Michalet-Doreau et al., 2001). Other estimates have put the *Fibrobacter* SSU DNA at 2.2 percent of total SSU DNA in the rumen (Ziemer et al., 2000). *Fibrobacter* spp. are tightly attached adherent bacteria, so it is possible that our extraction procedures were of limited effectiveness in terms of removing this bacterium from plant particles. A potential way to overcome this shortfall is that due DNA extraction from solids and to perform similar analysis. Another possibility is PCR basis due to the RIS primers used that competitively inhibited. However this does not appear to be the case, because both RIS primer had perfect matches to the 16S and 23S in *Fibrobacter succinogenes*. It would appear that *Fibrobacter* spp. were a relatively small percentage of the bacteria recovered.

Ruminococcus spp., especially *Ruminococcus albus* and Ruminococcus *flavefaciens,* are the other group of cellulolytic bacteria commonly isolated from the rumen. These bacteria were most readily identified in the libraries prepared from the adherent fractions, in both hay- and grain-fed animals, meaning these bacteria contribute to fiber degradation under both scenarios. Clones representing other Ruminococci, including *Ruminococcus hydrogenotrophicus* and *Ruminococcus schinkii* were also identified in the clone libraries. These bacteria differ from the cellulolytic Ruminococci species in that their predominant activity is to convert hydrogen and carbon dioxide to acetate (Bernalier et al., 1996 and Rieu-Lesme et al., 1996).

Prevotella species, such as *Prevotella bevis*, *Prevotella ruminicola* and others considered close to the *Bacteroides* spp. are numerically dominant in the 16S DNA libraries that are created from whole rumen samples. In this study, they accounted for less than 27 percent (except L C-2 at 46.8 percent), much less than is normally seen. *Prevotella/Bacteroides* are starch degrading bacteria capable of tolerating relative acidic conditions (pH <6.0), which is commonly observed in grain-fed animals. Hence, it is not surprising that the relative abundance of clones representing these groups was more common in the liquid fractions of grain-fed animals There still was a fair number of Prevotella in the adherent hay fractions which leads to the possibility of *Prevotella* being able to adhere to plant material. *Prevotella* can adhere to teeth and the possibility of a cellulose binding domain has been reported. *Mitsuokella jalaludinii* and related species are similar to Prevotella in substrate preferences. *Mitsuokella* utilizes glucose, celluboise and starch as a carbohydrate source (Lan et al., 2002) and were most numerous in the AD C-1 sample. Interestingly, *Mitsuokella* spp. has been shown to produce high levels of phytatase, an enzyme that is capable of breaking down phytate, a compound that binds phosphorus. This requires the feeding of higher levels of phosphorus and can increases losses in manure and runoff. As *Mitsuokella* was higher in the C-1 animal it could affect the levels of phosphorus that is available to the animal.

The Selenomonads are another important group of bacteria capable of degrading of soluble carbohydrates (Ricke et al., 1996). Selenomonads are very numerous in the rumen, especially *Selenomonas ruminantium*, and are a very diverse group in terms of

function and genetic makeup. Another species common in high concentrate diets is *Anaerovibrio lipolytica*. This species has been implicated in the role of lipolytic activity (Prins et al., 1975) and is highest in the concentrate fractions. *Anaerovibrio* is also important in its role of lactate fermentation, which helps to moderate the pH levels in the rumen (Dennis et al., 1981). Therefore, it is not surprising to find the libraries from grain-fed animals to contain a relatively large percentage of clones representing these bacterial genera.

It is widely accepted that many bacteria in the rumen can only function in a consortium with others. A good example is how *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium* are able to grow on pure xylan cultures (Cotta et al., 1995). The *Selenomonas ruminantium* are able to use the products of extracellular xylanolytic enzymes from *Butyrivibrio fibrisolvens*. Another example of the synergistic relationships that might exist among rumen bacteria may have been identified in these studies. Cellulose degradation by *Ruminococcus albus* is stimulated when phenylacetic acid (PAA) and phenylproponic acid (PPA) are available, although the microbial metabolic schemes and the bacteria involved with their production are yet to be identified. Several of the clones from my adherent libraries are a best match to sequences obtained from Mogibacterium *diversum* and *Eubacterium brachy,* and both these bacteria are capable of producing PAA and PPA (Nakazawa et al., 2002). My findings now allow the development of experiments with culturable isolates of *Mogibacterium or Eubacterium* spp. with *R. albus,* to determine whether these bacteria can produce PPA and PAA in the rumen that increases fiber degradation by *Ruminococcus albus*.

5. Concluding Remarks

I used a combination of RIS-LP and DGGE to examine bacterial diversity present in different fractions of ruminal digesta, and found that while differences between animals were evident, the techniques did not differentiate adequately between the different fractions of digesta collected. A combination of RIS-RFLP and DNA sequencing however revealed that the digesta fractions do indeed contain a high degree of diversity. These studies will serve as the foundation for creating a rumen microbe database so that in the future, RISA-RFLP patterns can be collected from other studies and used to speciate rumen microbes. My studies also revealed that in hay-fed animals especially, the Clostridia may play a more important role in fiber degradation than originally considered. Additionally, I was also able to identify candidate bacteria that may play a role in one of the most important, but undefined syntrophic relationships in the rumen: the production of PAA and PPA which are essential for efficient cellulose hydrolysis by *Ruminococcus albus*. More work needs to be done to identify and characterize these species and to quantify their overall effect on rumen ecology and function.

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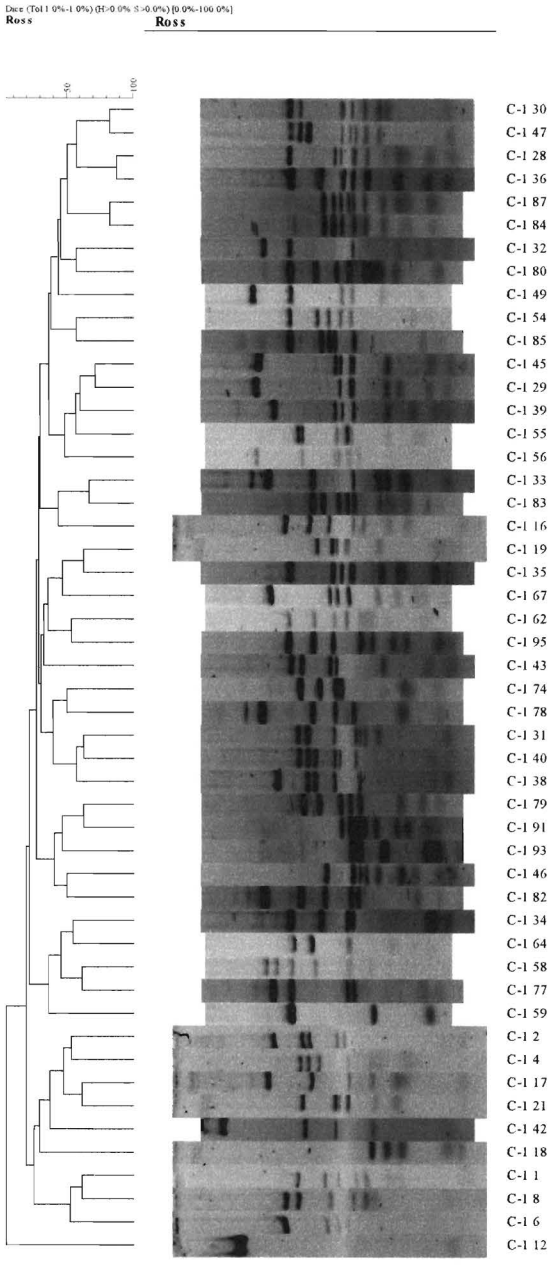
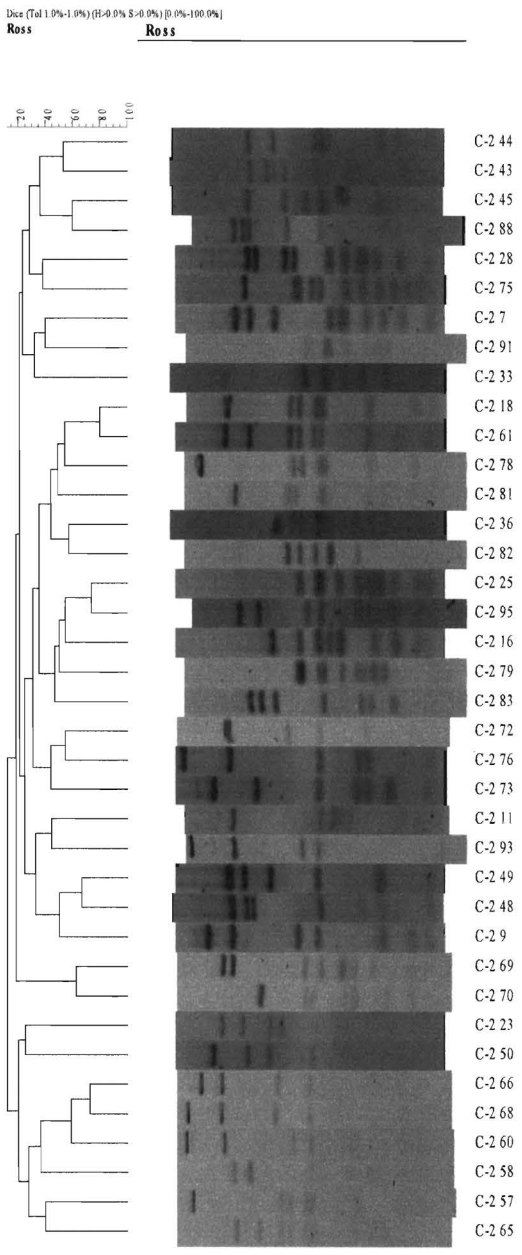
Appendix A: These dendograms, created with the BioNumerics program and Dice alignment, show the unique phylotypes of each fraction. Double digest of each RIS region were run on 3% agarose gels and viewed with ChemiImager 6600.

Adherent C-1 Library

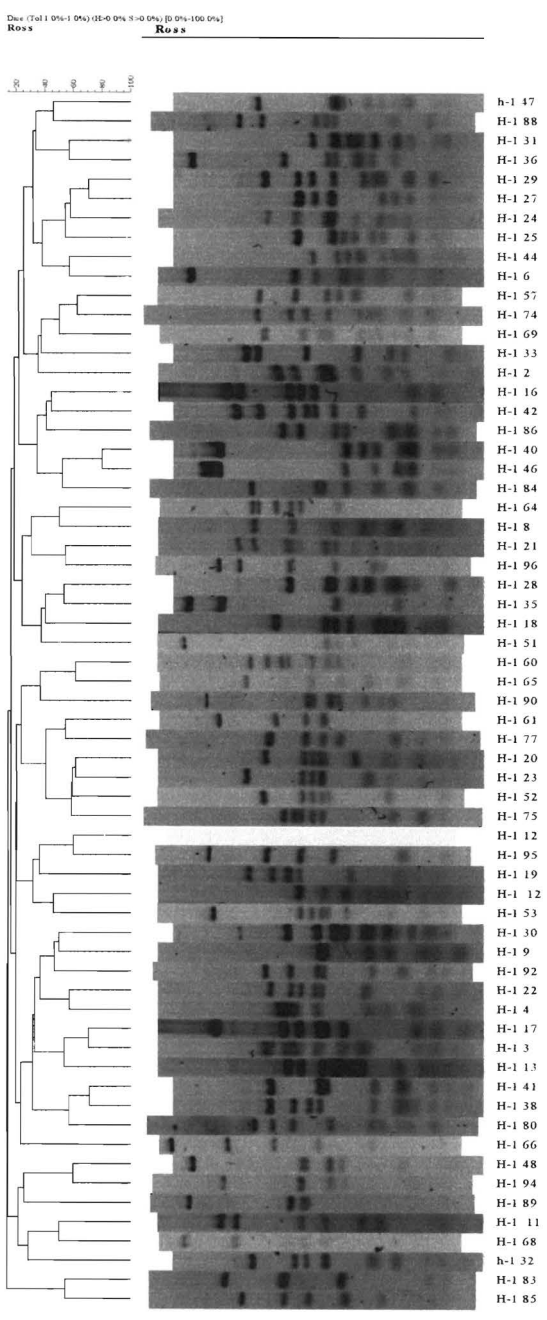


Adherent C-2 Library

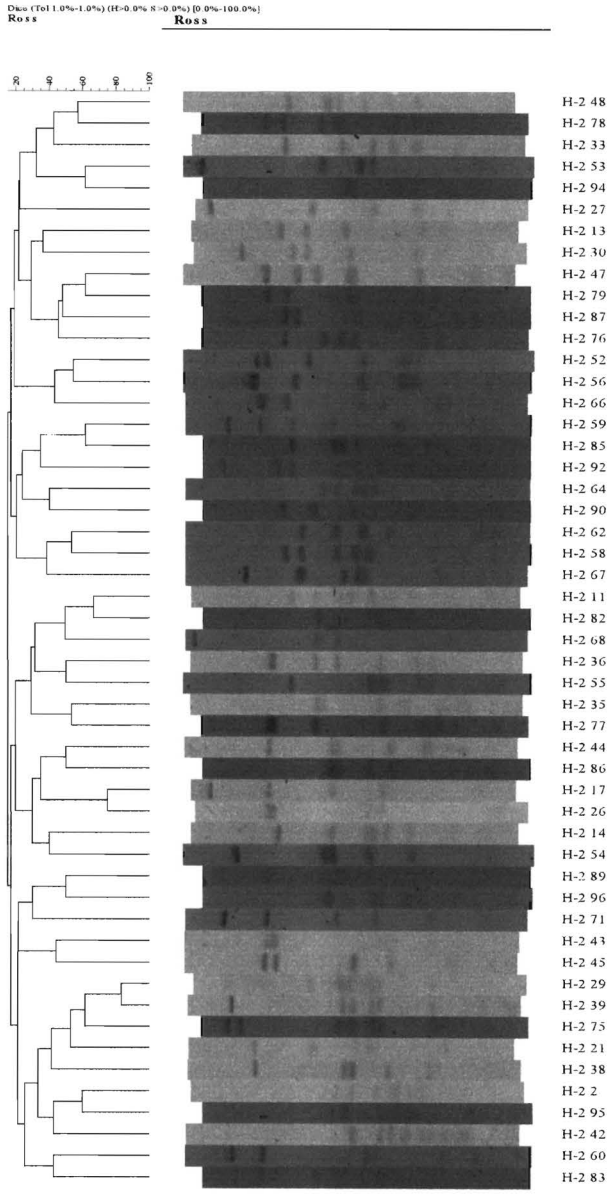


Appendix A cont.

Adherent H-1 Library

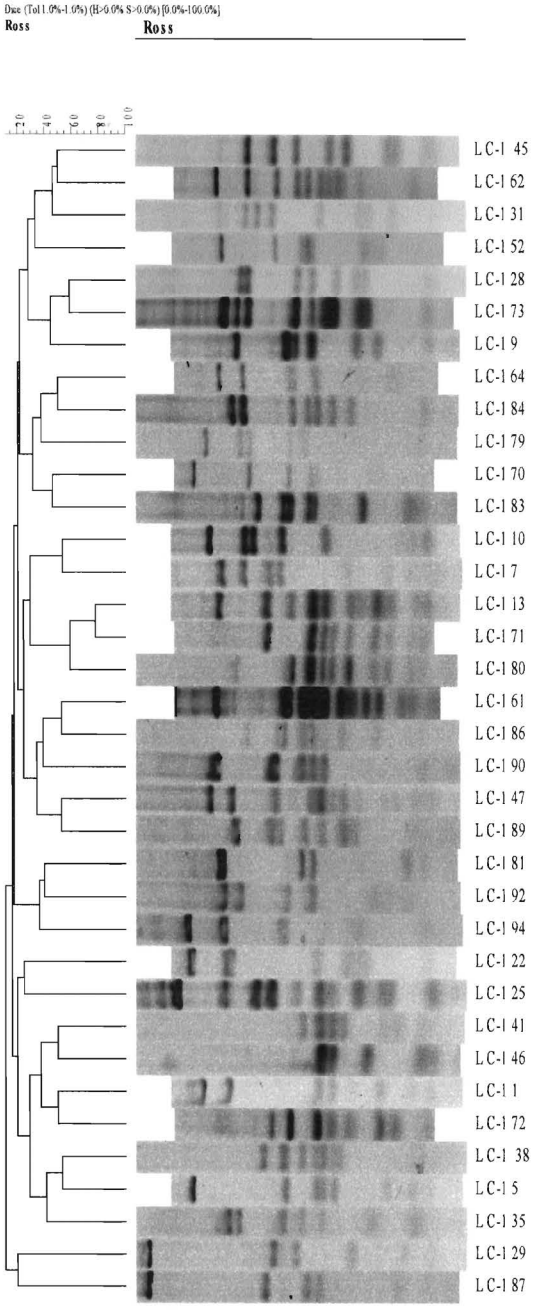


Adherent H-2 Library

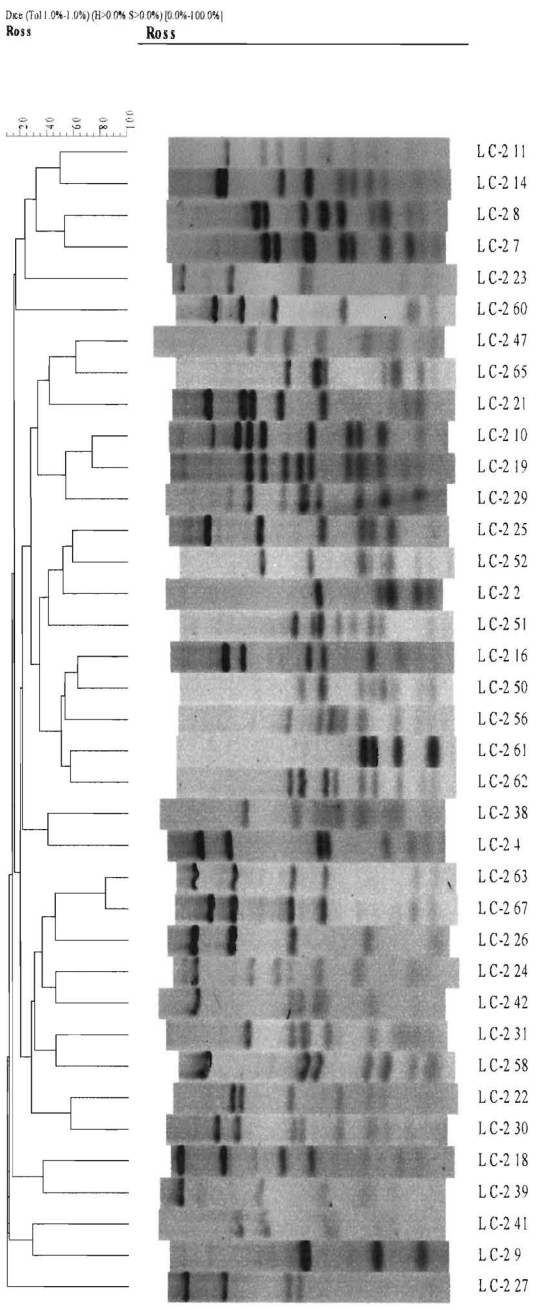


Appendix A cont.

Liquid C-1 Library

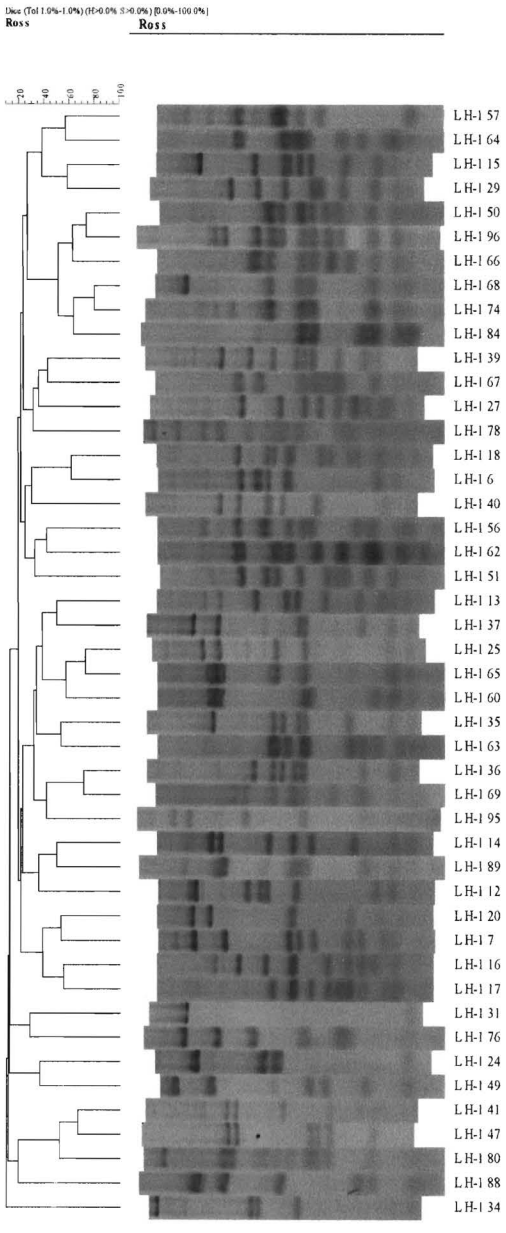


Liquid C-2 Library



Appendix A cont.

Liquid H-1 Library



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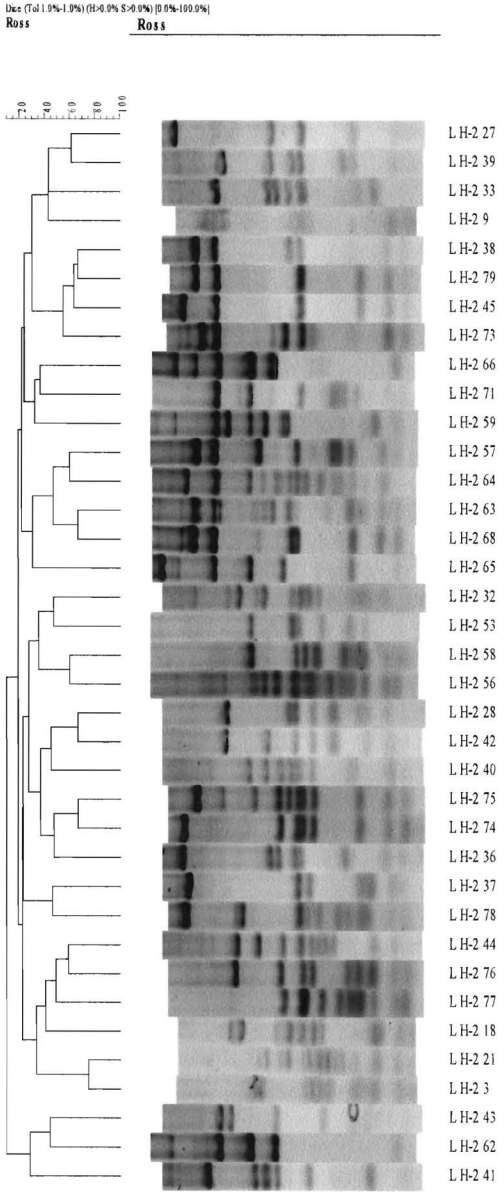

Appendix B: The list of microbes identified in the RIS libraries prepared from community DNA samples collected from liquid and adherent fractions of rumen digesta samples, based on 16S rDNA gene sequence alignments. In each case, the best match between the cloned sequence and those available in the databases are shown, and if the best match is with an "unidentified bacterium", then the best match with a known microbe is also shown.

Library	Total Number	Accession Number	Match	Score	Percent Match
AD C-1 1	1	AF371547.1	Uncultured bacteria	611	89%
		X89970.1	Butyrivibrio fibrisolvens	591	88%
AD C-1 2	6	AJ409004.1	Uncultured bacterium (colon)	747	91%
		U77341.1	Butyrivibrio fibrisolvens	708	90%
AD C-1 4	1	AB034084.1	Uncultured rumen bacterium	981	96%
		AF287794.1	Selenomonas (oral)	722	92%
AD C-1 6	1	AJ010959.1	Anaerovibrio lipolytica	1092	97%
AD C-1 8	1	AF371910.1	Unidentified rumen bacteria	634	93%
		AF139524.1	Bacteroides sp.	504	89%
AD C-1 12	1	AB034138.1	Uncultured rumen bacterium	989	96%
		AF479674.1	Mitsuokella jalaludinii	934	95%
AD C-1 16	1	AB034054.1	Uncultured rumen bacterium	823	92%
		X85101.1	Ruminococcus obeum	611	90%
AD C-1 17	1	AB034084.1	Uncultured rumen bacterium	989	96%
		AF287794.1	Selenomonas sp.	743	93%
AD C-1 18	1	AF133139.1	Pseudomonas	515	94%
AD C-1 19	3	AB009182.1	Unidentified rumen bacterium	1084	99%
		XB1137.1	Succiniclasticum ruminis	987	95%
AD C-1 21	1	AB034073.1	Uncultured rumen bacterium	1098	98%
		X85099.1	Ruminococcus bromii	995	96%
AD C-1 28	1	AB034081.1	Uncultured rumen bacterium	1140	99%
		AF479674.1	Mitsuokella julaludinii	981	95%
AD C-1 29	4	AB009182.1	Unidentified rumen bacterium	1096	99%
		X81137.1	Selenomonas ruminis	1009	96%
AD C-1 30	1	AF001737.1	Unidentified rumen bacterium	785	92%
		AF287792.1	Selenomonas like sp.	716	92%
AD C-1 31	1	AJ409004.1	Uncultured bacterium	765	91%
		X89975.1	Butyrivibrio fibrisolvens	716	90%
AD C-1 32	1	AB034054.1	Uncultured rumen bacterium	888	93%
		X94964.1	Ruminococcus schinkii	626	88%
AD C-1 33	1	AB034138.1	Uncultured rumen bacterium	1001	96%
		AF479674.1	Mitsuokella julaludinii	946	95%

AD C-1 34	1	AF357557.1	Bacteria mpn isolate	1088	99%
		AF479674.1	Mitsuokella julaludinii	979	96%
AD C-1 35	1	AF167711.1	Papillibacter cinnaminovorans	589	91%
AD C-1 36	1	AB034138.1	Uncultured rumen bacteria	852	93%
		AB017195.1	Selenomonas ruminantium	842	93%
AD C-1 38	12	AJ409004.1	Uncultured bacteria	765	91%
		U7734.1	Butyrivibrio fibrisolvens	722	90%
AD C-1 39	1	AB009231.1	Unidentified rumen bacterium	1007	97%
AD C-1 40	1	AJ409004.1	Uncultured rumen bacteria	730	91%
		AJ428552.1	Clostridium proteoclasticium	680	91%
AD C-1 42	1	AF233586.1	Methanobacterium congolense	765	91%
AD C-1 43	1	AB034082.1	Uncultured rumen bacteria	1146	99%
		AJ010959.1	Anaerovibrio lipolytica	1021	96%
AD C-1 45	2	AB009182.1	Unidentified rumen bacteria	1067	98%
		Z81137.1	Selenomonas ruminis	985	96%
AD C-1 46	7	AB009225.1	Unidentified rumen bacteria	965	96%
		AJ011682.1	Prevotella bevis	961	95%
AD C-1 47	1	AJ409004.1	Uncultured bacterium	726	90%
		U77341.1	Butyrivibrio fibrisolvens OB251	700	90%
AD C-1 49	1	AB034137.1	Uncultured rumen bacteria	1039	98%
		AF479674.1	Mitsuokella jalaludinii	906	97%
AD C-1 54	2	AJ010959.1	Anaerovibrio lipolytica	1098	97%
AD C-1 55	1	AB034006.1	Uncultured rumen bacteria	854	93%
		Z89971.1	Butyrivibrio fibrisolvens	741	91%
AD C-1 56	1	AB034073.1	Uncultured rumen bacteria	886	99%
		X85099.1	Ruminococcus bromii	767	96%
AD C-1 58	1	AB034059.1	Uncultured rumen bacteria	1126	98%
		AJ428553.1	Butyrivibrio hungatei	628	92%
AD C-1 59	1	AF403181.1	Desulfitobacterium hafniense	84	84%
AD C-1 62	1	AF371583.1	Uncultured bacterium	652	90%
		X95624.1	Ruminococcus hydrogenotrophicus	569	88%
AD C-1 64	1	AB034084.1	Uncultured rumen bacteria	983	96%
		AF287794.1	Selenomonas sp. (oral)	743	93%
AD C-1 67	2	AB009231.1	Unidentified rumen bacteria	918	95%
		AB021159.1	Bacteroides acidofaciens	468	87%
AD C-1 74	1	AF395430.1	Uncultured rumen bacteria	918	95%
		X76161.1	Clostridium aminobutyricum	862	93%
AD C-1 76	2	AB034003.1	Uncultured rumen bacteria	720	95%
		AF030446.1	Ruminococcus flavefaciens	652	89%
AD C-1 77	3	AB034138.1	Uncultured rumen bacteria	858	93%
		AF479674.1	Mitsuokella jalaludinii	842	92%
AD C-1 78	1	AB034084.1	Uncultured rumen bacteria	1154	99%
		AF3287794.1	Selenomonas sp. (oral)	735	92%
AD C-1 79	1	AB009225.1	Unidentified rumen bacteria	981	96%
		AJ011682.1	Prevotella brevis	934	95%
AD C-1 80	1	AF371583.1	Uncultured bacteria	652	90%

		X95624.1	Ruminococcus hydrogenotrophicus	569	88%
AD C-1 82	1	AB034185.1	Uncultured rumen methanogen	1132	99%
		AJ009958.1	Methanobrevibacter	1031	98%
AD C-1 83	2	AB034084.1	Uncultured rumen bacteria	720	95%
		AF287794.1	Selenomonas sp.	722	92%
AD C-1 84	1	AB034003.1	Uncultured rumen bacteria	720	95%
		AF104844.1	Ruminococcus flavefaciens	624	89%
AD C-1 85	1	AJ010959.1	Anaerovibrio lipolytica	1070	97%
AD C-1 87	2	AB034003.1	Uncultured rumen bacteria	733	95%
		AF104844.1	Ruminococcus flavefaciens	630	89%
AD C-1 91	5	AB056708.1	Uncultured equine intestine	438	91%
		AF414115.1	Flavobacteriaceace	291	85%
AD C-1 93	1	AF371572.1	Uncultured bacteria	426	89%
		AF157058	Eubacterium plexicaudatum	424	88%
AD C-1 95	1	AB039054.1	Uncultured rumen bacteria	898	94%
		X94964.1	Ruminococcus schinkii	636	88%
AD C-2 7	2	AF001737.1	Unidentified rumen bacteria	876	97%
		AF287794.1	Selenomonas sp. (oral)	712	92%
AD C-2 9	4	AF001706.1	Unidentified rumen bacterium	1029	97%
		Y18187.1	Clostridium orbiscindens	823	92%
AD C-2 11	1	AF001747.1	Unidentified rumen bacterium	839	93%
		AF481205.1	Bacteroidales (oral)	502	88%
AD C-2 16	2	AB003385.1	Prevotella	1039	98%
AD C-2 18	2	AB009182.1	Unidentified rumen bacteria	1057	98%
		X81137.1	Selenomonas ruminis	969	95%
AD C-2 28	1	AB034057.1	Uncultured rumen bacterium	973	96%
		AF298663.1	Lachnobacterium bovis	648	93%
AD C-2 43	1	AJ408993.2	Uncultured bacterium	842	93%
		AF126687.1	Clostridium fimetarium	817	92%
AD C-2 44	1	AJ408228.1	Uncultured equine intestine	777	92%
		AY005054.1	Prevotella sp. (oral)	492	90%
AD C-2 45	1	AJ408228.1	Uncultured equine intestine	777	92%
		AF218620.1	Prevotella ruminicola strain TF2-5	460	88%
AD C-2 48	1	AF030450.1	Ruminococcus flavefaciens	1102	99%
AD C-2 49	1	AF030450.1	Ruminococcus flavefaciens	1037	98%
AD C-2 50	1	AF371646.1	Uncultured bacterium clone	686	95%
		AF262239.1	Clostridium leptum	668	94%
AD C-2 57	2	AB009182.1	Unidentified rumen bacteria	1082	99%
		X81137.1	Selenomonas ruminis	985	96%
AD C-2 58	1	Af001737.1	Unidentified rumen bacteria	1035	98%
		AF287794.1	Selenomonas sp.	775	93%
AD C-2 60	1	AB034081.1	Uncultured rumen bacterium	1158	99%
		AF479674.1	Mitsuokella jalaludinii	999	96%
AD C-2 61	1	AF001773.1	Unidentified rumen bacteria	724	95%
		AB003386.1	Prevotella	692	94%

AD C-2 65	1	AJ408094.1	Uncultured equine intestine	1043	98%
		X85100.1	Ruminococcus callidus	692	90%
AD C-2 66	2	AB034031.1	Uncultured rumen bacterium	1102	98%
		AB017195.1	Selenomonas ruminantium	1102	98%
AD C-2 68	1	AB017195.1	Selenomonas ruminantium	930	95%
AD c-2 69	1	AJ408228.1	Uncultured equine intestine	805	93%
		AY005054.1	Prevotella sp. (oral)	492	90%
AD C-2 70	1	AJ408228.1	Uncultured equine intestine	777	92%
		AF218620.1	Prevotella ruminicola	460	88%
AD C-2 72	1	AF030450.1	Ruminococcus flavefaciens	1102	99%
AD C-2 75	1	AF030450.1	Ruminococcus flavefaciens	1037	98%
AD C-2 76	1	AF287794.1	Selenomonas sp.	741	92%
AD C-2 79	9	AF371646.1	Uncultured bacteria clone	686	95%
		AF262239.1	Clostridium leptum	668	94%
AD C-2 81	1	AB009182.1	Uncultured rumen bacteria	1082	99%
		X81137.1	Selenomonas ruminis	985	96%
AD C-2 83	2	AB034084.1	Uncultured rumen bacteria	995	96%
		AF287794.1	Selenomonas sp.	743	93%
AD C-2 88	2	AJ408228.1	Uncultured equine intestine	813	93%
		AY005054.1	Prevotella sp.	509	89%
AD C-2 91	3	AF218619.1	Prevotella sp.	934	95%
AD C-2 93	1	AF001753.1	Unidentified rumen bacteria	1031	98%
		AJ006963.1	Fusobacterium sulci	644	92%
AD C-2 95	1	AF371830.1	Uncultured bacterium	737	93%
		Y18180.1	Clostridium thermosuccinogenes	444	87%
AD H-1 2	1	AB056708.1	Uncultured equine intestine	438	91%
		AF414115.1	Flavobacteriaceace	291	85%
AD H-1 3	2	AF001737.1	Unidentified rumen bacteria	1035	98%
		AF287794.1	Selenomonas sp. (oral)	?	93%
AD H-1 4	1	AB009228.1	Unidentified rumen bacteria	1023	97%
		Y18180.1	Clostridium thermosuccinogenes	432	89%
AD H-1 6	2	AF371800.1	Uncultured bacterium clone p-2053	622	90%
		AF167711.1	Papillibacter cinnaminovorans	599	90%
AD H-1 8	1	AF371875.1	Uncultured bacterium clone p-219	948	96%
		AF218619.1	Prevotella ruminicola strain TC2-28	902	94%
AD H-1 9	1	AF001707.1	Unidentified rumen bacteria	730	93%
		AF481205.1	Bacteroides	458	90%
AD H-1 11	1	AF371933.1	Uncultured bacterium clone p-5013	726	92%
		Y18187.1	Clostridium orbiscindens	515	88%
AD H-1 12	1	AF001714.1	Unidentified rumen bacteria	1033	98%
		AF481228.1	Prevotella sp.	868	93%
AD H-1 13	1	AJ009933.1	Prevotella aff. ruminicola	983	95%

AD H-1 14	1	AF001733.1	Unidentified rumen bacteria	1055	98%
		AF481208.1	Clostridiales oral clone	710	90%
AD H-1 16	1	AB034010.1	Uncultured rumen bacteria	1005	97%
		AB037974.3	Mogibacterium diversum	783	92%
AD H-1 17	1	AB034012.1	Uncultured rumen bacteria	1063	97%
		Y18180.1	Clostridium thermosuccinogenes	464	90%
AD H-1 19	1	AF376145.1	Uncultured bacteria	761	92%
		L04165.1	Clostridium aminophilum	753	95%
AD H-1 20	1	U82327.1	Moorella glycerini	416	89%
AD H-1 21	1	AB009228.1	Unidentified rumen bacteria	698	89%
		Y18180.1	Clostridium thermosuccinogenes	305	83%
AD H-1 22	3	AB034012.1	Uncultured rumen bacteria	1094	98%
		U82327.1	Moorella glycerini	400	88%
AD H-1 23	1	AB009218.1	Unidentified rumen bacteria	1088	98%
		U82327.1	Moorella glycerini	408	88%
AD H-1 25	1	AB003386.1	Prevotella sp.	1084	99%
AD H-1 27	2	AB009231.1	Unidentified rumen bacteria	1023	97%
		AB021159.1	Bacteroides acidofaciens	482	88%
AD H-1 28	1	AB034028.1	Uncultured rumen bacteria	848	93%
		AF157049.1	Lactobacillus murinus	462	87%
AD H-1 29	3	AB034102.1	Uncultured rumen bacteria	973	96%
		AF218619.1	Prevotella ruminicola	946	95%
AD H-1 30	1	AJ408228.1	Uncultured equine intestine	624	89%
		AB021159.1	Bacteroides acidofaciens	402	89%
AD H-1 31	2	AB034100.1	Uncultured rumen bacteria	898	95%
		AB003386.1	Prevotella sp.	870	94%
AD H-1 32	5	AB009218.1	Unidentified rumen bacteria	1088	98%
		U82327.1	Moorella glycerini	408	88%
AD H-1 33	3	AF371830.1	Uncultured bacterium clone	801	94%
		Y18180.1	Clostridium thermosuccinogenes	490	88%
AD H-1 35	1	AJ408190.1	Uncultured equine intestine	787	92%
		Y11466.1	Holdemania filiformis	688	91%
AD H-1 38	1	AF371830.1	Uncultured bacterium	809	94%
		Y18180.1	Clostridium thermosuccinogenes	490	88%
AD H-1 36	1	AF371800.1	Uncultured bacterium	622	90%
		AF167711.1	Papillibacter cinnaminovorans	607	90%
AD H-1 40	1	AF050544.1	Uncultured eubacterium	743	92%
		AF385564.1	Prevotella sp.	595	91%
AD H-1 41	1	AF371830.1	Uncultured bacterium	932	96%
		Y18180.1	Clostridium thermosuccinogenes	474	88%
AD H-1 42	2	AF332710.1	Uncultured bacteria	932	96%
		X85099.1	Ruminococcus bromii	890	94%
AD H-1 44	3	AF001714.1	Unidentified rumen bacteria	1041	98%
		AF218619.1	Prevotella ruminicola	936	95%
AD H-1 46	1	AF050544.1	Uncultured equine intestine	743	92%
		AF385564.1	Prevotella	595	91%
AD H-1 47	2	AJ408123.1	Uncultured equine intestine	666	94%
		AB021161.1	Bacteroides acidofaciens	595	91%
AD H-1 48	1	AF424491.1	Uncultured planetomycete	688	90%

		X81952.1	Planctomyces	662	89%
AD H-1 51	1	AJ400254.2	Uncultured bacterium	605	91%
		AF481226.1	Prevotella	547	90%
AD H-1 52	1	AF287773.1	Firmicutes	793	92%
AD H-1 53	1	AB034012.1	Uncultured rumen bacteria	1068	97%
		Y18180.1	Clostridium thermosuccinogenes	464	90%
AD H-1 57	1	AF001747.1	Unidentified rumen bacteria	916	95%
		AF481207.1	Bacteroidales	609	91%
AD H-1 60	1	AF371886.1	Uncultured bacteria	767	92%
		AF218619.1	Prevotella ruminicola	527	94%
AD H-1 61	1	AB034073.1	Uncultured rumen bacteria	912	95%
		X859099.1	Ruminococcus bromii	904	94%
AD H-1 64	1	AF371666.1	Uncultured bacteria (pig)	726	94%
		AJ409000.1	Clostridium	686	90%
AD H-1 65	1	AF371875.1	Uncultured bacteria (pig)	932	96%
		AF218619.1	Prevotella ruminicola	904	94%
AD H-1 66	1	AF001753.1	Unidentified rumen bacteria	1047	98%
		AF287761.1	Eubacterium sp.	656	93%
AD H-1 68	1	AF001706.1	Unidentified rumen bacteria	896	94%
		Y18187.1	Clostridium orbiscindens	688	89%
AD H-1 74	2	AB034102.1	Uncultured rumen bacteria	981	96%
		AF218619.1	Prevotella ruminicola	946	95%
AD H-1 75	2	AB034023.1	Uncultured rumen bacteria	1031	96%
		Y18180.1	Clostridium thermosuccinogenes	424	89%
AD H-1 77	2	AF220064.1	Bulleidia extructa	916	94%
AD H-1 80	2	AJ408056.1	Uncultured equine intestine	880	94%
		AF218618.1	Prevotella ruminicola	799	92%
AD H-1 83	1	AF371875.1	Uncultured bacteria (pig)	987	97%
		AF432140.1	Firmicutes	579	91%
AD H-1 84	1	AF371875.1	Uncultured bacteria (pig)	948	96%
		AF218619.1	Prevotella ruminicola	914	94%
AD H-1 85	1	AF371875.1	Uncultured bacteria (pig)	985	97%
		AF432140.1	Firmicutes	543	90%
AD H-1 86	1	AF001714.1	Unidentified rumen bacteria	944	97%
		AF040719.1	Prevotella	789	94%
AD H-1 88	1	AB034138.1	Uncultured rumen bacteria	914	94%
		AB017195.1	Selenomonas ruminantium	914	95%
AD H-1 89	1	AB034003.1	Uncultured rumen bacteria	1011	97%
		AJ305238.1	Clostridium leptum	731	90%
AD H-1 90	1	AB034003.1	Uncultured rumen bacteria	1011	97%
		AJ305238.1	Clostridium leptum	731	90%
AD H-1 92	1	AF371830.1	Uncultured bacteria (pig)	809	94%
		Y18180.1	Clostridium thermosuccinogenes	474	88%
AD H-1 94	1	AF050568.1	Uncultured eubacterium	846	93%
		AF424392.1	Uncultured green nonsulfur bacteria	626	88%
AD H-1 95	2	AB034084.1	Uncultured rumen bacteria	1110	99%
		AF287794.1	Selenomonas sp.	700	92%
AD H-1 96	1	AF001733.1	Unidentified rumen bacteria	1055	98%
		AF481208.1	Clostridiales sp.	710	90%

AD H-2 11	1	X81876.1	Prevotella dentalis	686	89%
AD H-2 13	1	AJ408156.1	Uncultured equine intestine	579	88%
		AF292372.1	Atopobium parvulum (oral)	563	89%
AD H-2 17	1	ABo34147.1	Uncultured rumen bacterium	715	91%
		AJ310135.1	Eubacterium pyruvovarans	549	89%
AD H-2 26	1	AF201986.1	Uncultured human clone	872	93%
		AF287794.1	Selenomonas sp.	842	93%
AD H-2 27	1	AB034028.1	Uncultured rumen bacterium	797	95%
		AY036904.1	Desulfotomaculum kuznetabuii	400	88%
AD H-2 29	1	AB009182.1	Unidentified rumen bacteria	1104	99%
		X81137.1	Selenomonas ruminis	1023	96%
AD H-2 30	1	AF001700.1	Unidentified rumen bacteria	1035	97%
		X89974.1	Butyrivibrio fibrisolvens	751	91%
AD H-2 33	2	AB034012.1	Uncultured rumen bacteria	1084	97%
		Y18180.1	Clostridium thermosuccinogenes	448	90%
AD H-2 35	2	AF001714.1	Unidentified rumen bacteria	1102	99%
		AF218619.1	Prevotella ruminicola	868	93%
AD H-2 38	1	AF001770.1	Unidentified rumen bacteria	854	93%
		AF432140.1	Firmicutes sp.	474	88%
AD H-2 44	2	AF001702.1	Unidentified rumen bacteria	946	96%
		X71858.1	Clostridium polysaccharolyticum	688	89%
AD H-2 45	3	AB034147.1	Uncultured rumen bacteria	770	94%
		X76161.1	Clostridium aminobutyricum	615	94%
AD H-2 47	8	AF001700.1	Unidentified rumen bacteria	1082	98%
		X89974.1	Butyrivibrio fibrisolvens	751	91%
AD H-2 48	1	AB034032.1	Uncultured rumen bacteria	854	93%
		AF481221.1	Lachnospiraceae	718	94%
AD H-2 52	4	AF001733.1	Unidentified rumen bacteria	1070	98%
		AF481208.1	Clostridiales (oral)	704	90%
AD H-2 53	1	AF001746.1	Unidentified rumen bacteria	801	92%
		AB021159.1	Bacteroides acidofaciens	498	88%
AD H-2 54	1	AF001770.1	Unidentified rumen bacteria	1021	97%
		AF432140.1	Firmicutes (oral)	539	90%
AD H-2 55	1	AB045744.1	Uncultured fiber-attached rumen bacteria	914	98%
		AB003387.1	Prevotella	817	95%
AD H-2 56	1	AF001733.1	Unidentified rumen bacteria	1070	98%
		AF481208.1	Clostridales sp.	720	90%
AD H-2 58	1	X85099.1	Ruminococcus bromii	817	94%
AD H-2 59	1	Af371631.1	Uncultured bacterium	789	93%
		V41168.1	Butyrivibrio fibrisolvens	773	92%
AD H-2 60	2	Af371830.1	Uncultured bacterium	779	94%
		Y18180.1	Clostridium thermosuccinogenes	456	88%
AD H-2 62	2	AB009211.1	Unidentified rumen bacteria	781	94%
		AF218618.1	Prevotella rumincola strain 223/M2/7	563	90%
AD H-2 66	1	AB034011.1	Unidentified rumen bacteria	1051	97%
		AF167711.1	Papillibacter cinnaminovorans	876	93%

AD H-2 67	1	AF079847.1	Ruminococcus albus	987	96%
AD H-2 75	1	AB034047.1	Uncultured rumen bacteria	311	94%
		L09187.1	Clostridium fervidus	204	90%
AD H-2 76	1	AF371528.1	Uncultured bacteria (pig)	753	92%
		AF085350.1	Mycoplasma mycoides	375	83%
AD H-2 77	7	AF001733.1	Unidentified rumen bacteria	765	92%
		AF481208.1	Clostridiales (oral)	460	85%
AD H-2 78	2	AF001714.1	Unidentified rumen bacteria	908	96%
		AB003386.1	Prevotella	777	94%
AD H-2 79	1	AB009195.1	Unidentified rumen bacteria	977	97%
		U68426.2	Chlamydophila pneumoniae	369	84%
AD H-2 82	1	AF001714.1	Unidentified rumen bacteria	928	96%
		AB003401.1	Prevotella ruminicola	864	94%
AD H-2 83	2	AF079847.1	Ruminococcus albus	904	93%
AD H-2 85	1	AB034032.1	Uncultured rumen bacteria	799	92%
		AF481221.1	Lachnospiraceae	702	93%
AD H-2 86	2	AJ318130.1	Uncultured bacteria (waste biofiliter)	367	91%
		AF027010.1	Cytophagales/green sulfur	319	88%
AD H-2 87	3	AF371830.1	Uncultured bacterium (pig)	777	94%
		Y18180.1	Clostridium thermosuccinogenes	498	88%
AD H-2 89	1	AB034032.1	Uncultured rumen bacteria	886	94%
		AF481221.1	Lachnospiraceae	726	94%
AD H-2 90	1	AF371830.1	Uncultured bacteria	793	94%
		Y18180.1	Clostridium thermosuccinogenes	498	88%
AD H-2 92	4	AF030446.1	Ruminococcus flavefaciens	759	96%
AD H-2 94	1	AF050568.1	Uncultured eubacterium	793	92%
		AF424402.2	Uncultured green nonsulfur bacteria	618	88%
AD H-2 96	5	AB009227.1	Unidentified rumen bacteria	789	93%
		AJ251195.1	Paenibacillus azotofix	432	89%
L C-1 1	5	AB034081.1	Uncultured rumen bacteria	1112	98%
		AF479674.1	Mitsuokella julaludinii	954	95%
L C-1 5	1	AF287794.1	Selenomonas (oral)	898	94%
L C-1 7	1	AJ270482.2	Unidentified butyrate degrader	640	93%
		AF202259.1	Eubacterium oxidoreducens	597	91%
L C-1 10	2	AJ409004.1	Uncultured bacterium	757	91%
		AJ428552.1	Clostridium proteoclasticum	708	90%
L C-1 13	2	AF372875.1	Uncultured bacteria	646	92%
		AF218619.1	Prevotella ruminicola	622	91%
L C-1 25	1	AF001737.1	Unidentified rumen bacteria	981	97%
		AF287794.1	Selenomonas sp.	743	93%
L C-1 28	2	AJ409004.1	Uncultured bacterium	763	91%
		AJ428552.1	Clostridium proteoclasticum	714	91%
L C-1 29	1	AB034185.1	Uncultured rumen methanogen	1120	99%
		AJ009958.1	Methanobrecibacter sp.	1023	98%

L C-1 35	1	AJ010959.1	Anaerovibrio lipolytica	1090	97%
L C-1 38	1	AF371571.1	Uncultured bacteria	648	90%
		X89976.1	Butyrivibrio fibrisolvens	642	91%
L C-1 41	1	AB009225.1	Unidentified rumen bacteria	967	96%
		AJo11682.1	Prevotella bevis	920	94%
L C-1 45	1	AF371547.1	Uncultured bacteria	611	89%
		X89970.1	Butyrivibrio fibrisolvens	607	88%
L C-1 46	2	AB009225.1	Unidentified rumen bacteria	827	96%
		AF218619.1	Prevotella ruminicola	787	95%
L C-1 47	1	AB034138.1	Uncultured rumen bacteria	678	90%
		AF221598.1	Selenomonas ruminantium	670	90%
L C-1 52	3	AJ408228.1	Uncultured equine bacteria	801	93%
		AF218620.1	Prevotella ruminicola	482	88%
L C-1 53	1	AJ409004.1	Uncultured rumen bacteria	579	87%
		U77341.1	Butyrivibrio fibrisolvens	565	87%
L C-1 58	1	AB009225.1	Unidentified rumen bacteria	981	96%
		AJ611682.1	Prevotella bevis	940	95%
L C-1 61	1	AB009225.1	Unidentified rumen bacteria	973	96%
		Aj011682.1	Prevotella bevis	948	95%
L C-1 62	1	AF371583.1	Uncultured bacteria	652	90%
		X95624.1	Ruminococcus hydrogenotrophicus	569	88%
L C-1 64	1	AB034138.1	Uncultured rumen bacteria	829	93%
		AB017195,1	Selenomonas ruminantium	827	93%
L C-1 70	1	Y09434.1	Schwartzia succinivorans	807	94%
L C-1 71	18	AB009225.1	Unidentified rumen bacteria	957	96%
		AJ011682.1	Prevotella bevis	940	95%
L C-1 72	1	AJ409004.1	Uncultured rumen bacteria	648	89%
		U77341.1	Butyrivibrio fibrisolvens	618	88%
L C-1 73	1	AJ270473.2	Unidentified butyrate degrader	714	92%
		AF124902.1	Butyrivibrio fibrisolvens	682	90%
L C-1 79	1	AJ409004.1	Uncultured bacterium (colon)	763	91%
		AJ428552.1	Clostridium proteoclasticum	714	91%
L C-1 80	8	AB009225.1	Unidentified rumen bacteria	965	96%
		AJ011682.1	Prevotella bevis	954	95%
L C-1 81	3	AF201986.1	Uncultured human bacteria	858	93%
		AF287794.1	Selenomonas sp.	837	93%
L C-1 83	2	AB009201.1	Unidentified rumen bacteria	1019	97%
		X81137.1	Selenomonas ruminis	1015	97%
L C-1 84	1	AJ409004.1	Uncultured bacterium	731	91%
		AJ428552.1	Clostridium proteoclasticum	698	90%
L C-1 86	3	Y17600.1	Succinivibrio dextrinosolvens	1116	99%
L C-1 87	1	AB034185.1	Uncultured rumen methanogen	1112	99%
		AJ009958.1	Methanobrecibacter	1921	98%
L C-1 89	1	AF371910.1	Uncultured bacteria (pig)	634	93%
		AF139524.1	Bacteroides	478	88%
L C-1 90	1	AF201986.1	Uncultured human bacteria	858	93%
		AF479674.1	Mitsuokella jalaludinii	850	93%
L C-1 92	1	AB034138.1	Uncultured rumen bacteria	967	95%

		AF479674.1	Mitsuokella jalaludinii	904	94%
L C-1 94	1	AB034082.1	Uncultured rumen bacteria	1096	98%
		AJ010959	Anaerovibrio lipolytica	1009	96%
L C-2 2	3	AF050568.1	Uncultured eubacterium	618	87%
		AF424402.1	Unidentified green nonsulfur bacteria	444	83%
L C-2 4	2	AF371830.1	Uncultured bacterium	777	94%
		Y18180.1	Clostridium thermosuccinogenes	466	88%
L C-2 7	2	AF371875.1	Uncultured bacterium	884	94%
		AJ011682.1	Prevotella ruminicola	876	93%
L C-2 8	1	AB003385.1	Prevotella	1005	97%
L C-2 9	1	AB056643.1	Uncultured fiber attaching rumen bacteria	765	96%
		AF218619.1	Prevotella ruminicola	716	93%
L C-2 10	1	AB034084.1	Uncultured rumen bacteria	952	96%
		AF287794.1	Selenomonas	708	92%
L C-2 11	2	AB034003.1	Uncultured rumen bacteria	668	94%
		AF030446.1	Ruminococcus flavefaciens	587	88%
L C-2 14	2	AF371772.1	Uncultured bacterium	1076	99%
		X85099.1	Ruminococcus bromii	963	96%
L C-2 16	1	AF001706.1	Unidentified rumen bacteria	1023	97%
		Y18187.1	Clostridium orbiscindens	827	92%
L C-2 18	1	AF371700.1	Uncultured bacteria	1023	98%
		AB017195.1	Selenomonas ruminantium	1015	97%
L C-2 19	1	AB034084.1	Uncultured rumen bacteria	989	96%
		AF287794.1	Selenomonas	743	93%
L C-2 21	1	AJ409004.1	Uncultured bacteria	757	91%
		AJ428552.1	Clostridium proteoclasticum	708	90%
L C-2 22	1	AF371830.1	Uncultured bacteria	777	94%
		Y18180.1	Clostridium thermosuccinogenes	438	89%
L C-2 23	2	AB034007.1	Uncultured rumen bacteria	1015	97%
		Z36272.1	Eubacterium brachy	797	93%
L C-2 24	1	X76164.1	Clostridium longisporum	1102	98%
L C-2 25	3	AB034084.1	Uncultured rumen bacteria	975	96%
		AF287794.1	Selenomonas	730	92%
L C-2 26	1	AB034082.1	Uncultured rumen bacteria	1108	98%
		AJ010959.1	Anaerovibrio lipolytica	1021	96%
L C-2 27	1	AB034010.1	Uncultured rumen bacteria	1035	97%
		AB037874.1	Mogibacterium diversum	797	92%
L C-2 29	1	AJ408194.1	Uncultured equine intestine	1053	98%
		Y18187.1	Clostridium orbiscindens	936	95%
L C-2 30	1	AB034059.1	Uncultured rumen bacteria	741	95%
		AJ428553.1	Butyrivibrio hungatei	460	89%
L C-2 31	1	AB034057	Uncultured rumen bacteria	1112	99%
		AF287773.1	Firmicutes	632	92%
L C-2 38	1	AB034003.1	Uncultured rumen bacteria	728	95%
		AF104844.1	Ruminococcus flavefaciens	628	89%
L C-2 39	1	AF001737.1	Unidentified rumen bacteria	924	96%

		AF287794.1	Selenomonas (oral)	793	94%
L C-2 41	1	AB034027.1	Uncultured rumen bacteria	387	94%
		X75909.1	Clostridium sp.	329	91%
L C-2 47	1	AJ270473.2	Unidentified butyrate	730	92%
		AJ428552.1	Clostridium proteoclasticum	678	91%
L C-2 51	4	AF371583.1	Uncultured bacteria	555	88%
		AF385577.1	Catonella	531	86%
L C-2 56	1	AB009180.1	Unidentified rumen bacteria	1019	98%
		AB003385.1	Prevotella sp.	940	96%
L C-2 58	1	AF371513.1	Uncultured bacteria	963	97%
		AJ241721.1	Clostridium innocuum	551	90%
L C-2 60	2	No good			
L C-2 61	7	No good	Too short		
L C-2 62	4	AF018477.1	Unidentified rumen bacteria	1049	99%
		AB003386.1	Prevotella sp.	755	93%
L C-2 63	1	AB017195.1	Selenomonas ruminantium	1112	99%
L C-2 67	2	AF001774.1	Unidentified rumen bacteria	1096	99%
		AB017195.1	Selenomonas ruminantium	1070	98%
L C-2 65	1	AB034102.1	Uncultured rumen bacteria	938	95%
		AF218619.1	Prevotella	914	94%
L H-1 6	2	AF001765.1	Unidentified rumen bacteria	747	91%
		AF385563	Eubacterium sp.	587	91%
L H-1 7	1	AJ408259.1	Uncultured equine intestine	617	94%
		AF385555.1	Porphyromonas sp.	563	87%
L H-1 12	1	AF001737.1	Unidentified rumen bacteria	807	95%
		AF287794.1	Selenomonas	730	93%
L H-1 13	1	AB009223.1	Unidentified rumen bacteria	961	96%
		AF218619.1	Prevotella ruminicola	955	95%
L H-1 16	3	AB017195.1	Selenomonas ruminantium	1088	99%
L H-1 17	4	AF544206.1	Rumen bacteria	926	95%
		AF218619.1	Prevotella ruminicola	906	94%
L H-1 18	1	AB009227.1	Unidentified rumen bacteria	924	95%
		Y18180.1	Clostridium thermosuccinogenes	424	89%
L H-1 20	1	AB017195.1	Selenomonas ruminantium	1070	98%
L H-1 24	2	AB034016.1	Uncultured rumen bacteria	692	93%
		X94230.1	Lactobacillus panis	353	91%
L H-1 27	5	AB034012.1	Uncultured rumen bacteria	942	95%
		Y18180.1	Clostridium thermosuccinogenes	440	89%
L H-1 29	2	AB003385.1	Prevotella	1051	98%
L H-1 31	1	AB009182.1	Unidentified rumen bacteria	1072	99%
		X81137.1	Selenomonas ruminis	983	96%
L H-1 34	1	AB034016.1	Uncultured rumen bacteria	908	94%
		X94230.1	Lactobacillus panis	329	90%
L H-1 36	3	AB003385.1	Prevotella	870	97%

L H-1 37	1	AB034031.1	Uncultured rumen bacteria	1051	98%
		AB017195.1	Selenomonas ruminantium	1051	97%
L H-1 39	1	AF129861.1	Uncultured bacteria (digestor)	922	96%
		AF167711.1	Papillbacter cinnaminovans	827	92%
L H-1 40	2	AF371628.1	Uncultured bacteria	882	94%
		U77339.1	Butyrivibrio fibrisolvens	846	93%
L H-1 44	6	AB009225.1	Unidentified rumen bacteria	852	96%
		AF218619.1	Prevotella	813	95%
L H-1 47	3	AF371830.1	Uncultured bacteria	775	93%
		Y18180.1	Clostridium thermosuccinogenes	466	87%
L H-1 49	1	AF371528.1	Uncultured bacteria	496	87%
		X76331.1	Lactobacillus sanfrancisco	238	85%
L H-1 50	1	AF018440.1	Unidentified rumen bacteria	368	89%
		AF218619.1	Prevotella ruminicola	287	94%
L H-1 57	1	AF001714.1	Unidentified rumen bacteria	900	96%
		AB003386.1	Prevotella	769	94%
L H-1 60	2	AF201986.1	Uncultured human bacteria	858	93%
		AF287794.1	Selenomonas sp.	842	93%
L H-1 62	1	AF371875.1	Uncultured bacteria	704	92%
		AF218620.1	Prevotella ruminicola	644	91%
L H-1 64	1	AJ408139.1	Uncultured equine bacteria	620	89%
		AJ305238.1	Clostridium leptum	553	90%
L H-1 65	2	AF201986.1	Uncultured human bacteria	815	93%
		AF287793.1	Selenomonas sputigena	807	92%
L H-1 66	1	AB003385.1	Prevotella	995	97%
L H-1 67	1	AB009180.1	Unidentified rumen bacteria	1039	98%
		AB003385.1	Prevotella	963	96%
L H-1 68	1	AF371893.1	Uncultured bacterium (pig)	751	93%
		L16475.1	Prevotella bivia	739	92%
L H-1 69	1	AF371528.1	Uncultured bacteria	817	93%
		AJ006775.1	Spiroplasma	408	85%
L H-1 74	2	No good	Multiple bands		
L H-1 76	1	AJ408217.1	Uncultured equine intestine	432	89%
		U40791.1	Spirochaeta	176	87%
L H-1 78	1	AB009182.1	Unidentified rumen bacteria	1035	99%
		X81137.1	Selenomonas ruminis	934	96%
L H-1 80	1	AF371830.1	Uncultured rumen bacteria	587	88%
		Y18180.1	Clostridium thermosuccinogenes	329	84%
L H-1 84	1	NO good			
L H-1 88	2	AF371830.1	Uncultured bacterium	785	94%
		Y18180.1	Clostridium thermosuccinogenes	468	88%
L H-1 89	1	AB009188.1	Unidentified rumen bacteria	985	97%
		AB003379.1	Selenomonas ruminantium	985	97%
L H-1 95	1	AB034084.1	Uncultured rumen bacteria	987	99%
		AF287794.1	Selenomonas sp. (oral)	694	92%
L H-2 18	2	AF018497.1	Unidentified rumen bacteria	817	95%

		AF218619.1	Prevotella ruminicola	507	94%
L H-2 21	1	AB003385.1	Prevotella	975	98%
L H-2 27	1	AB009193.1	Unidentified rumen bacteria	868	95%
		AB003390.1	Bacteroides	448	89%
L H-2 28	1	AB034013.1	Unidentified rumen bacteria	993	96%
		Y18180.1	Clostridium thermosuccinogenes	448	90%
L H-2 32	1	AB034138.1	Uncultured rumen bacteria	811	92%
		AF287794.1	Selenomonas sp.	795	92%
L H-2 33	1	AF030448.1	Ruminococcus flavefaciens	1055	97%
L H-2 35	1	AJ408094.1	Uncultured equine intestine	1009	97%
		X85099.1	Ruminococcus bromii	573	91%
L H-2 36	1	AB009182.1	Unidentified rumen bacteria	1063	99%
		X81137.1	Selenomonas ruminis	979	96%
L H-2 37	1	AJ408123.1	Uncultured equine intestine	603	94%
		L16485.1	Bacteroides erggerthii	509	91%
L H-2 38	2	AF371703.1	Uncultured bacteria	789	93%
		AB017195.1	Selenomonas ruminantium	781	92%
L H-2 39	1	AB09193.1	Unidentified rumen bacteria	890	95%
		AB003390.1	Bacteroides	450	89%
L H-2 40	1	AF201986.1	Uncultured human (oral)	825	92%
		AF479674.1	Mitsuokella jalaludinii	817	92%
L H-2 41	1	M59112.1	Clostridium symbiosum	799	93%
L H-2 42	1	AB034012.1	Uncultured rumen bacteria	1021	96%
		Y18180.1	Clostridium thermosuccinogenes	448	90%
L H-2 44	2	X81876.1	Prevotella dentalis	634	90%
L H-2 45	1	AB034138.1	Uncultured rumen bacteria	967	96%
		AF479674.1	Mitsuokella jalaludinii	936	95%
L H-2 53	1	AF419688.1	Uncultured bacteria	285	90%
		AB015525.1	Cytophaga	246	87%
L H-2 56	5	AF001773.1	unidentified rumen bacteria	852	94%
		AF218619.1	Prevotella ruminicola	825	92%
L H-2 59	1	AJ408208.1	Uncultured equine intestine	718	92%
		AF044945.1	Eubacterium sp.	622	90%
L H-2 62	3	AF001741.1	unidentified rumen bacteria	591	92%
		AF432140.1	Firmicutes	317	85%
L H-2 63	1	AB034138.1	Uncultured rumen bacteria	228	87%
		AB017195.1	Selenomonas ruminantium	228	87%
L H-2 64	1	AF001733.1	unidentified rumen bacteria	611	87%
		AF481208.1	Clostridiales (oral)	242	94%
L H-2 65	1	AF132255.1	Uncultured bacteria	690	91%
		AF167711.1	Papillibacter cinnaminovorans	589	88%
L H-2 66	1	No good			
L H-2 68	3	AB017195.1	Selenomonas	1096	98%
L H-2 71	2	AF001733.1	Unidentified rumen bacteria	1017	97%
		AF481208.1	Clostridiales (oral)	670	90%
L H-2 73	4	AB017195.1	Selenomonas ruminantium	1021	97%

L H-2 74	1	AB009182.1	Unidentified rumen bacteria	1051	98%
		X81137.1	Selenomonas ruminis	959	95%
L H-2 75	1	AB034013.1	Uncultured rumen bacteria	979	96%
		Y18180.1	Clostridium thermosuccinogenes	448	89%
L H-2 76	3	AF133139.1	Pseudomonas sp.	803	98%
L H-2 77	4	AF001773.1	Unidentified rumen bacteria	858	95%
		AF218619.1	Prevotella ruminicola	807	93%
L H-2 78	1	AF385497.1	Selenomonas specie (oral)	74	95%